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13. ABSTRACT (Maximum 200 Words) Fas/Fas ligand (FasL) is one of the key elements of the signaling pathway leading to cell death (apoptosis). It has been shown that FasL is overexpressed in some solid tumors and importantly, anticancer agents induce FasL expression in many tumor cell lines. Therefore, we have been using a mouse tumor model to investigate whether anticancer agents such as VP-16 induce FasL expression <i>in vivo</i> and whether FasL expression promotes the growth of Fas deficient tumor cells. Preliminary data with mouse mammary tumor cells (CRL-2116) support our hypothesis that anticancer drugs can induce FasL expression <i>in vivo</i> although more experiments are under way to confirm these results. To test the effect of drug-induced FasL expression on tumor growth, we will use CRL-2116 cells to construct a stable Fas ^{def} cell line that can be used as a negative control. Thus, we have cloned the wild-type mouse Fas and its dominant negative Fas mutant, and confirmed that both clones are expressed exogenously. Once a stable Fas ^{def} CRL-2116 cell line is obtained, we will examine the effect of the drug-induced FasL on tumor growth of the Fas ^{def} CRL-2116 cells.				
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Role of drug-induced Fas ligand expression in breast tumor progression

Annual report

(4) Introduction

Chemotherapeutic agents kill cancer cells by multiple mechanisms, including intercalation into DNA, inhibition of DNA replication or cell membrane damage. Although the primary intracellular targets of drug action are rather distinct, it has become evident that drug-induced cytotoxicity ultimately converges on a common pathway, causing apoptosis. Drug-induced cytotoxicity involves proteases of the caspase family because specific inhibitors of caspases prevent cell death after treatment with different anticancer agents. One of the best defined apoptotic pathways is mediated by the surface receptor Fas, a member of the TNF receptor superfamily (1, 2). Triggering of the receptor by its natural ligand, FasL, or agonistic antibodies induces the formation of a death-inducing signaling complex and then activates down stream caspases (3). It has been previously shown that FasL is induced by anticancer agents as well as other DNA damaging agents (4). An example is induction of FasL by topoisomerase inhibitors (e.g., VP-16 and doxorubicin) (4, 5). These agents cause DNA damage to cells by stabilizing broken DNA-enzyme complexes. However, the role of drug-induced FasL expression is controversial. For instance, it has been reported that in some leukemia cell lines, FasL induced by the anticancer drugs is responsible for apoptosis of the tumor cells because neutralizing Fas antibody is able to block the apoptosis (4). By contrast, others have shown that the anticancer agents cause cell death independent of FasL induction (6, 7). Nevertheless, these reports all confirmed that the anticancer agents induce FasL expression. We have shown that DNA damage appears to signal this FasL induction (8). Thus, all of these *in vitro* studies demonstrate a role for anticancer agents in the induction of FasL, but it is not clear whether the drugs induce FasL *in vivo*. If FasL is induced *in vivo*, what is the effect of FasL induction on tumor and host immune system?

Fas/FasL is one of major surface proteins of T lymphocytes and plays an important role in immune defense and homeostasis of the immune system (1). It is well known that activated T lymphocytes exert cytotoxicity against tumor cells or undergo apoptosis to eliminate excess activated T lymphocytes through Fas/FasL interaction (3). However, previous studies have shown that many types of tumor cells overexpress FasL while Fas expression is down-regulated, suggesting that tumor cells might escape cytotoxic activity of host immune T-lymphocytes through different expression levels of Fas/FasL (9-11). Indeed, it has been shown in several cases that FasL produced by tumor cells is capable of killing T lymphocytes (12, 13). If anticancer agents induce FasL expression in tumor cells that are defective in the Fas pathway, then the induced FasL might be able to be used as a weapon against T lymphocytes. Therefore, we proposed to examine if FasL is induced by these agents *in vivo*. If yes, is there any effect on tumor growth? Because of an important role of Fas/FasL in the immune system, we believe that induction of FasL will have a significant effect on tumor growth. Our approach is to use a tumor xenograft model as an initial step towards characterization of FasL induction by anticancer drugs, and then to use mouse mammary cancer cells to investigate the effect of such FasL induction on tumor growth *in vivo*.

(5) Body

Our project did not start until April of 2000, because the research specialist hired to work on this project was not able to come to the lab until April 1, 2000 (see budget sheet). Thus, the schedule was a few months behind what we originally planned. However, in this short period, we have obtained some interesting preliminary data as described below.

I. Use mouse tumor model to test whether FasL is induced by anticancer agents in MCF-7 *in vitro* and *in vivo*

Pilot experiment testing for tumor growth in immune-deficient mice

We tested both nude mice and SCID mice. Both types of mice permit good tumor growth under the conditions described below. Based on the information available in the literature (14), we did some pilot experiments to test different aspects of tumor growth on these mice. These include varying tumor cell inoculum, total tumor cells, and amount of Metrigel in inoculation solution. We found the following combinations supported good tumor growth (Table 1).

Table 1. Tumor growth (MCF-7) on SCID mice under different conditions

Metrigel ^a	+	+	-	-
β -estradiol (0.72 mg)	-	+	-	+
Tumor growth	No	Yes	No	No

One and half million MCF-7 cells were inoculated per site.

^a50% Metrigel:50% PBS.

1. 50%:50% of Metrigel:tumor cells in PBS. Final concentration of tumor cells before injection was at 10 million cells/ml
2. Number of tumor cells for inoculation was 1.5 million cells per site in the mammary pad.
3. One pellet of β -estradiol (0.72 mg) was implanted underneath the back skin of the mouse.

Under these conditions, tumors became visible one week after inoculation (Fig. 1, right). Then, mice started to die after three weeks, which gave us a three-week window to work with. Since we did not observe significant differences between SCID mice and nude mice in terms of tumor growth, we used the SCID mice in the following experiments.

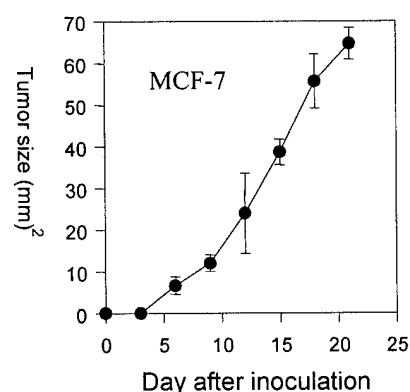


Fig. 1 Tumor growth of MCF-7 on SCID mice. The values are mean \pm SD of tumor size from five mice as measured on the days identified.

II. Initial experiments on SCID mice with MCF-7

Since VM-26 seems to be toxic to the animals, we used the less toxic and clinically used drug VP-16 (etoposide). To confirm that VP-16 is also able to induce FasL expression, we first examined FasL expression using MCF-7 cells *in vitro* in response to VP-16 treatment. As shown in Fig. 2A (right), VP-16 induced FasL expression in a dose-dependent manner. FasL expression level started to increase at 0.5 $\mu\text{g/ml}$, reached a maximal level at 5 $\mu\text{g/ml}$, and then started to decrease at 10 $\mu\text{g/ml}$. This result is similar to that for VM-26 (see the proposal).

We then tested the effect of VP-16 in the tumor-bearing SCID mice. So far we have tested a total of 70 mice. One week after inoculation, VP-16 was given as an i.p. injection at the following concentrations. In cases where more than one injection was given, the following injection was given one week after the first one. DMSO alone served as a negative control.

- 10 mice at 20 μg VP-16/g body weight with 1 injection (all tumors were recovered)
- 10 mice at 20 μg VP-16/g body weight with 2 injections (all tumors were recovered)
- 10 mice at 25 μg VP-16/g body weight with 1 injection (all tumors were recovered)
- 10 mice at 25 μg VP-16/g body weight with 2 injections (mice died; no tumors were recovered)
- 10 mice at 30 μg VP-16/g body weight with 1 injection (3 tumors were recovered)
- 10 mice at 30 μg VP-16/g body weight with 2 injections (mice died; no tumors were recovered)
- 10 mice at 50 μg VP-16/g body weight with 1 injection (mice died; no tumors were recovered)

The recovered tumors were quickly put on dry ice and then stored at -80°C before they were lysed for protein extraction. Protein concentrations were determined by using protein assay kit (Bio Rad) before separation on 11% SDS-PAGE. Preliminary results indicated no significant difference between drug-treated and non-treated tumors in expression of FasL. A representative Western blot is shown in Fig. 2B (above). However, since these are preliminary results, we need to do more experiments before we can draw any conclusions. It appears that the drug dose above 25 μg VP-16/g body weight is toxic to the animals. Thus, have decided to continue subsequent experiments using a dose of 20 $\mu\text{g/g}$ body weight.

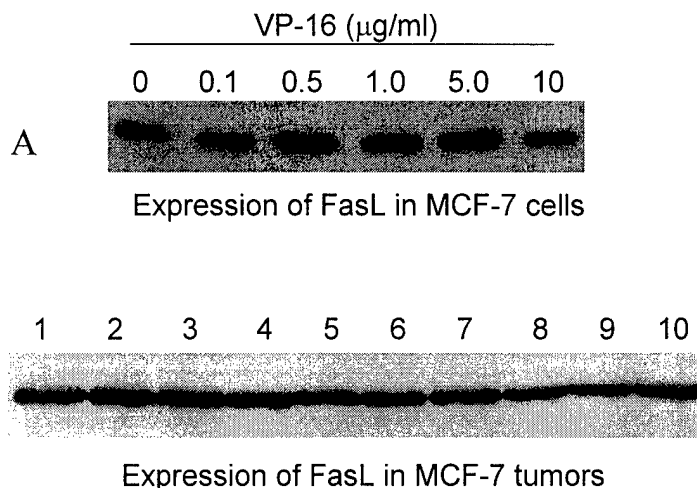


Fig. 2 Expression of FasL in MCF-7 cells and tumors as determined by Western blot. **A**, MCF-7 cells were treated with VP-16 at indicated concentrations for 24 h and then harvested for total protein extraction. **B**, MCF-7 cells were inoculated into SCID mice and VP-16 was administered one week after inoculation. Tumors were recovered after two injections of VP-16 (25 $\mu\text{g/g}$ body weight). Lanes 1-2, DMSO control; Lanes 3-10, VP-16-treated tumors.

III. Test FasL induction *in vivo* of mouse mammary tumor cells on wild-type mice and establish stable Fas^{def} mouse mammary tumor cell lines

This part of the project was planned for the second year of the research, but we started ahead of the plan.

One purpose of using mouse mammary tumor cell lines was to set up another way to test whether FasL is induced *in vivo* by VP-16. Eventually we can use these cells to test the effect of FasL expression on tumor growth. Since we are going to set up a mouse tumor model, we have to make sure that the cells will grow tumors in these mice. Thus, as proposed in the application, we first tested mouse mammary cells (FM3A) for tumor growth on wild-type mice. Unfortunately, FM3A cells appeared to be less tumorigenic than expected because they did not form tumors in the wild-type mice under the conditions described above. By contrast, another mouse mammary tumor cell line (CRL-2116) was much more tumorigenic under the same conditions (see below). Therefore, we switched to CRL-2116 cells for the subsequent experiments.

A. Characterization of CRL-2116 cells

Since there is little information in the literature about Fas/FasL expression and tumorigenicity in CRL-2116, we characterized these cells as follows:

1. Tumor development in wild-type mice

Using the same procedures for SCID mice, we found that CRL-2116 developed tumors relatively well in the wild-type mice (BALB/C) (Fig. 3, right). Tumors appeared one week after inoculation and all of the mice survived 4 weeks after inoculation. Thus, CRL-2116 cells are tumorigenic in these mice.

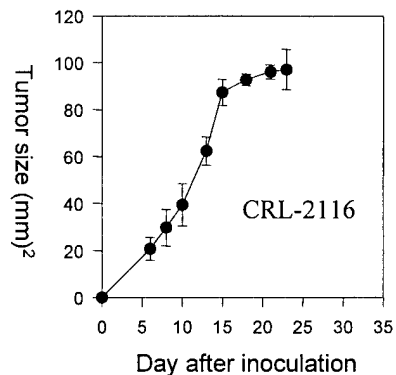


Fig. 3 Tumor growth of CRL-2116 on wild-type mice (C/BALB). Values are mean \pm SD tumor size from four mice.

2. Constitutive expression of Fas and FasL

In order to confirm that CRL-2116 cells carry an intact Fas pathway, we examined constitutive expression of Fas and FasL. RT-PCR demonstrated that CRL-2116 cells express both Fas and FasL (Fig. 4A, next page) although FasL expression (lane 5) is relatively low compared to that of mouse thymus cells (lane 3). Expression of FasL (Fig. 4B, next page and Fas (lane 3 of Fig. 6, page 9) was also confirmed by Western blot.

3. CRL-2116 cells are sensitive to killing by FasL.

Another way of determining if these cells retain an intact Fas pathway is to test if they are sensitive to killing by FasL. It has been demonstrated that FasL is able to kill wild-type Fas carrying cells. Jurkat cells were used here as a positive control. As shown in Fig. 4C (next page), FasL killed CRL-2116 cells although its sensitivity to FasL is relatively low compared to Jurkat cells, possibly because they are a different type of cell. Nevertheless, the results suggested that CRL-2116 cells have a functional Fas pathway.

4. FasL induction *in vitro* by VP-16.

Our ultimate goal is to test if drug-induced FasL has any effect on tumor growth. Therefore, we examined FasL expression *in vitro* after VP-16 treatment. As shown in Fig. 4B (above), FasL expression increased from 0 to 5 μ g VP-16 /ml and then decreased at 10 μ g/ml, suggesting that VP-16 can induce mouse FasL expression at least *in vitro*.

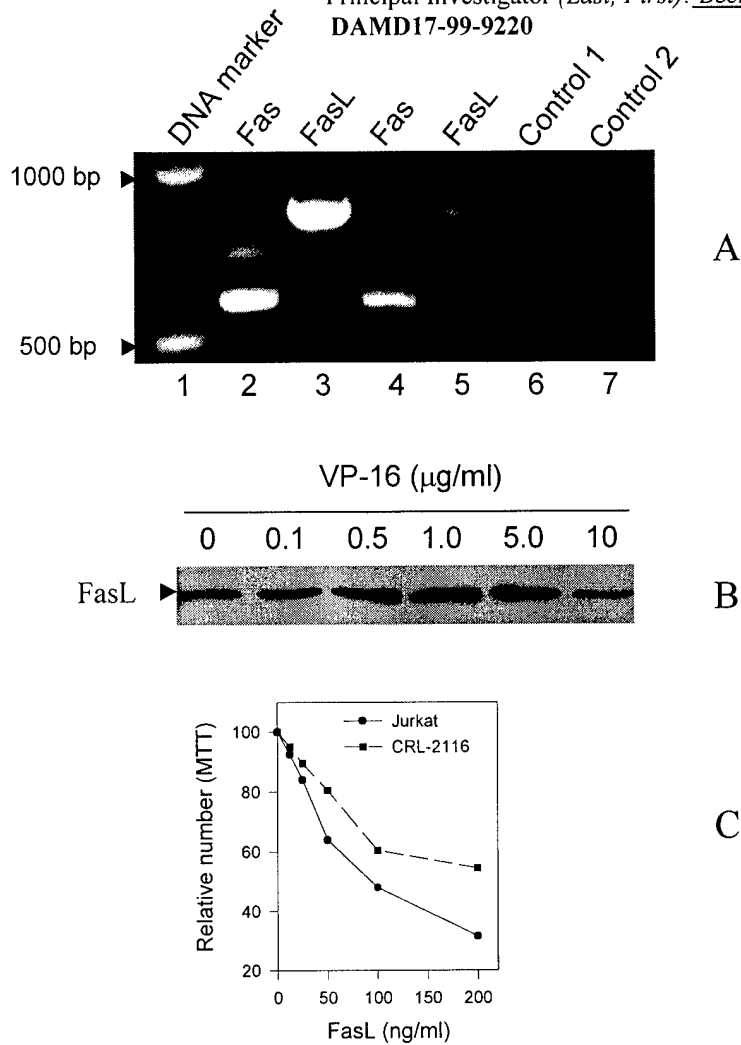


Fig. 4 Expression of Fas/FasL in CRL-2116 cells. **A**, RT-PCR detection of Fas/FasL. Lanes 2 and 3, mouse thymus; lanes 4 and 5, CRL-2116; lanes 6 and 7, negative controls for Fas and FasL, respectively. **B**, Expression of FasL *in vitro* in response to VP-16 by Western blot. **C**, Cell killing in response to FasL as measured by MTT assay. Values are average of two experiments.

5. Expression of FasL *in vivo* after VP-16 treatment

Using the same procedures as for SCID mice, We tested the effect of VP-16 on FasL expression. After 3 injections of VP-16 at 25 $\mu\text{g/g}$ body weight, tumors were removed, quickly frozen and kept at -80°C . RT-PCR revealed higher levels of mouse Fas mRNA in drug-treated tumors (lanes 2-6 in Fig.5, right) than that of no-drug-treated tumors (lane 1 in Fig. 5). Although more experiments are needed to confirm these findings with internal controls, the results seem to support the notion that

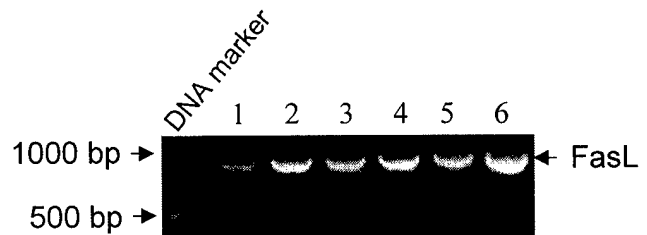


Fig. 5 RT-PCR detection of mouse FasL expression by CRL-2116 tumors in response to VP-16 treatment. Mice received three injections of VP-16 at 25 $\mu\text{g/g}$ body weight before tumors were removed, as described in the text. Lane 1, DMSO control; lanes 2-6, VP-16 treatment.

anticancer drugs can induce FasL expression *in vivo*.

B. Establishment of Fas defective mouse mammary tumor cell lines

As stated in the proposal, to determine if drug-induced FasL contributes to immune escape of tumor cells, we need to have an isogenic cell line that is defective in the Fas pathway for comparison. Since there is no such Fas deficient mouse cell line available, we were planning to make a Fas^{def} cell line by introducing a dominant Fas mutant. It has been shown that mutations (including deletions) in the death domain of Fas lead to blockage of the Fas pathway by FasL (15-17). This is the basis for us to make a Fas^{def} cell line.

1. Construction of expression plasmid that carries the dominant negative mouse Fas mutant.

The full-length mouse Fas consists of 326 aa that can be divided into three domains, the extracellular (aa1-159), the transmembrane (aa160-190) and the cytoplasmic domain (aa191-326) (see ref. 18 and Fig. 6A, right). The cytoplasmic domain is essential for Fas to recruit death components such as FADD and caspase-8. The dominant negative function of the Fas relies on its ability to interfere with the recruiting process because upon oligomerization, the deleted Fas molecule forms an incomplete complex with the endogenous wild-type Fas molecule. This incomplete complex is not able to initiate death signaling so the Fas pathway is blocked. Therefore, we deleted aa 197-326 i.e., we removed a majority of the cytoplasmic domain of Fas.

To make a deleted construct, we first amplified a deleted Fas as well as a full-length Fas cDNA from a wild-type mouse thymus by RT-PCR. The amplified fragments were cloned into pCR2.1 vector. For convenient subcloning, a restriction site (BamHI) was introduced at mFAS-5.1 and a EcoRI site was derived from the pRC2.1 vector. The sense primer mFAS-5.1 (GGATCCGCAGACATGCTGTGGATCTGGGGCT) and antisense primer mFAS-3.1 (AAGCTTCACTCCAGACATTGTCCTTC) were used to construct pMFAS-14; mFAS5.1 and mFAS-3.3 (TCAGCACTTTCTTTTCCGG) were used to construct pMFAS-15. Once sequences were verified by DNA sequencing using the Sanger method with Sequenase Version II (Amersham), these fragments were then cloned into pcDNA3 vector at BamHI and EcoRI sites.

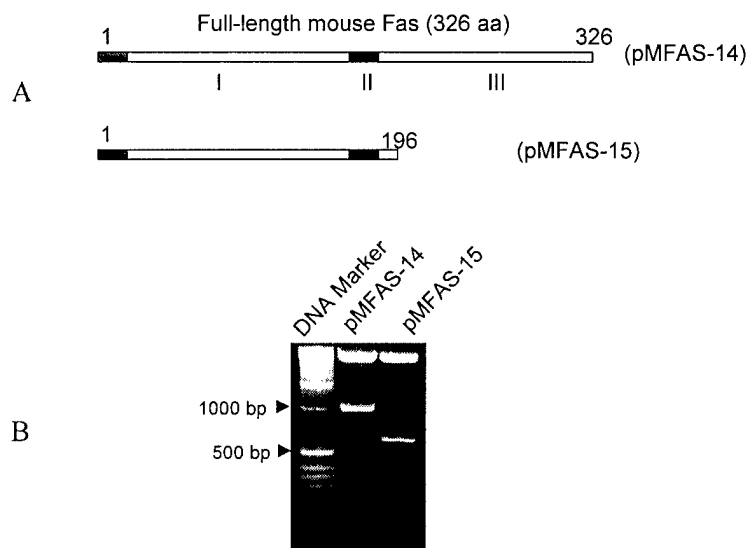


Fig. 6 Construction of mouse Fas plasmids in pCDNA3. **A**, Schematic description of mouse Fas plasmids. (I), extracellular domain (aa 21-167); (II), transmembrane domain (aa 168-186) and (III) cytoplasmic domain (aa 187-326). **B**, An agarose gel picture of clones pMFAS-14 and pMFAS-15 digested with both BamHI and EcoRI.

As shown in Fig. 6B (above), a 1000 bp fragment was released by digestion of EcoRI and BamHI from pMFAS-14 and a 600 bp fragment from pMFAS-15 DNA as expected.

2. Mouse Fas is expressed in transiently transfected HeLa cells

Expression of the exogenous mouse Fas was examined by Western blot of transiently transfected HeLa cells. Expression of the full-length construct (pMFAS-14) was relatively low compared to the deleted Fas (Fig. 7, right). We observed a slight increase of the 47 kDa band (lane 1, in Fig. 7), which co-migrated with the endogenous Fas. However, pMFAS-15 expressed a high level of protein band of about 15 kDa (lane 2 in Fig. 7). Compared to HeLa cells, CRL-2116 cells expressed a relatively high level of the endogenous Fas (lane 3 in Fig. 7).

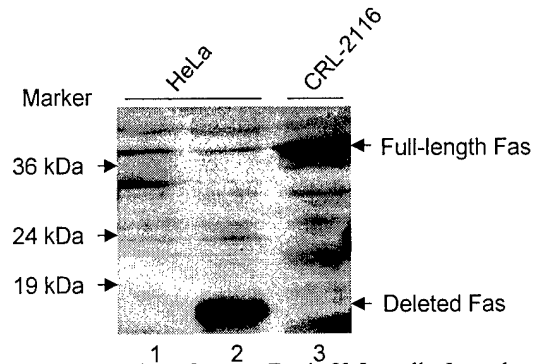


Fig. 7 Expression of mouse Fas in HeLa cells. Lane 1, pMFAS-14 (full-length mouse Fas); lane 2, pMFAS-15 (truncated mouse Fas); and lane 3, CRL-2116 cells. The blot was probed with rabbit anti-Fas antibodies that recognize both human and mouse Fas.

3. Establish stable cell lines

Right now we are in the process of selecting stable transfected cell lines that express the dominant negative Fas mutant. We expect that this will be complete in the next 2-3 months. Once the stable cell lines expressing the dominant negative Fas are obtained, we will first test their sensitivity to killing by FasL and then test for tumor growth on mice.

Future work for next year

We will follow the Statement of Work:

1. Test effect of VP-16 on FasL induction *in vivo*
2. Test effect of ICRF-187 on VP-16-induced FasL expression
3. Detect FasL promoter activity response to drugs *in vivo*
4. Establish stable CRL-2116 cell line expressing Fas dominant mutant

(6) Key research accomplishments

- ◆ We have constructed full-length and deleted mouse Fas expression plasmids that can be used to make Fas^{def} cell lines as well as other related research.
- ◆ Establishment of CRL-2116 cell lines expressing Fas dominant negative mutant is under way and is expected to be finished within 2-3 months.

(7) Reportable outcomes

- ◆ None yet.

(8) Conclusions

During the last 6 months, we have been working on the mouse tumor model to examine whether FasL is induced *in vivo* by the anticancer drug VP-16. Although we did not see a significant difference in FasL expression between VP-16 treatment and no drug

control in initial experiments with MCF-7 cells, preliminary data with CRL-2116 cells suggested that mouse FasL is induced by VP-16 *in vivo*. More experiments will be done in next a few months to confirm these findings. We have demonstrated that C2116-CLR cells express both Fas and FasL. Since these cells are sensitive to FasL killing, we believe that they carry a functional Fas/FasL pathway. Establishment of stable CRL-2116 cell line expressing dominant negative Fas will allow us to test whether there is any effect of drug-induced FasL on tumor growth.

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(10) Appendix

N/A